

Biliverdin: the blue-green pigment in the bones of the garfish (*Belone belone*) and eelpout (*Zoarces viviparus*)

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Abstract Little is known about the bluish-green color of the skeleton and bones of the garfish (*Belone belone*) and eelpout (*Zoarces viviparus*). Most of the few, contradictory reasons given favor either the iron(II) phosphate, vivianite, or the heme degradation product, biliverdin, but all lack documented spectroscopic confirmation. Our aim was to identify and quantify this blue-green substance and to investigate more precisely its location within the colored parts of these fish species. First, we found that vivianite was not indicated by the iron and phosphate levels in fish samples, whereas biliverdin was confirmed in the blue-green matrices both by UV–Vis (maxima at 376 and 666 nm) and FTIR spectroscopy, and by HPLC–MS/MS (molecular ion m/z $[M + H]^+ = 611$) after sample preparation involving solvent extraction, methylation (yielding biliverdin dimethyl ester), and solid-phase extraction. The overall average garfish biliverdin recovery rate by HPLC–UV was 72.3 %; the average biliverdin content of the vertebral column (including the periosteum) was 23.49 µg/g; periosteum, 61.70 µg/g; and spinal process (Processus spinosus), 24.3 µg/g. Analysis of amino acid distribution in colored and non-colored fish matrices conformed high proportions of hydroxyproline in the former (periosteum 7 %, spinal processes 7.6 %), suggesting that biliverdin has

a specific affinity for the structural protein collagen, and not for the bone itself. Finally, all methods confirmed the presence of biliverdin in collagen-rich tissues (periosteum and spinal processes) of garfish and eelpout, and we established a new method for the detection of biliverdin at low concentrations in structural proteins.

Keywords Blue-green pigment · Garfish (*Belone belone*) · Eelpout (*Zoarces viviparus*) · Biliverdin · Substance characterization (UV–Vis, FTIR, HPLC–MS) · Collagen

Introduction

The garfish is endemic to the North and Baltic Seas, the Mediterranean Sea, and the North Atlantic Ocean coastal waters of France, Spain, Portugal, and Morocco. In Germany, the garfish (Hornhecht) is also known as “green bone” (Grünknochen) or “horn fish.” The viviparous eelpout is found along the northeastern Atlantic coast and in the Baltic Sea. Both fish species are edible, but the eelpout is scarcely available commercially. Regionally, garfish plays an important role on the Island of Rügen, similar to that of brined herring (matjes) in the Netherlands.

The garfish (*Belone belone*) and eelpout (*Zoarces viviparus*) share a special feature: The skeleton, scales, and bones are conspicuously bluish-green. Contradictory theories have been proposed for the chemical compound responsible for this color, but no definitive spectroscopic identification has been made of the pigment. In the year 1941, Fontaine [1] assumed that carotenoids were responsible for the color of the garfish, and the blue-green tetrapyrrolic bile pigment biliverdin, a product of heme catabolism, was soon included in investigations: Wills-taedt [2] determined that raw extracts of garfish and

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eelpout in anhydrous acetic acid underwent the Gmelin bile pigment reaction and that even chromatographically purified fractions produced strong color reactions with nitric acid containing nitrites (Gmelin's test), leading to the suspicion that the pigments could be related to those of bile. However, Willstaedt ruled out biliverdin because the pigments he extracted, unlike the gall pigment biliverdin, were very instable in the presence of bases and could not be extracted from solution in ether into aqueous hydrochloric acid. For these reasons, he suggested heavy metal complexes as a possible explanation for the colors. He also was inclined to exclude products of carotinoid addition, almost exactly at the same time as Fontaine [1] had suggested these as a possibility. Pursuant to Willstaedt's positive identification of the bile pigments in 1940 [2], Çağlar [3] treated the extracted pigment in acidic environment with NaNO_2 and found that the garfish pigment changed in color from a blue-green to a purple-to-reddish hue. The same reaction was shown by control biliverdin, but no spectroscopic analyses were conducted that would have confirmed the identification by wet chemical methods. Willstaedt [2] conducted early simple spectroscopic analyses of the blue-green pigment, but the first correct, typical UV–Vis spectrum of the “garfish bone” pigment with two maxima at 385 and 675 nm was not made until 1957 [4], by Christomanos. However, on the basis of spectroscopic examinations and solubility tests, he concluded that the “green dyestuff” found in the scales of garfish was related to the bile pigments, but definitely different from biliverdin. On the other hand, Nomura and Tsuchiya [5] performed extraction on scales from garfish and the Pacific saury (*Cololabis saira*), which also belongs to the Beloniformes order, and compared the UV–Vis spectra of the colored extracts. Those investigators found that the absorption curves from the two fish species were almost identical and corresponded well to those published by Christomanos [4]. Unlike Christomanos, they assumed that the substance in question was very similar to biliverdin—but without comparison with a control substance.

Modern analytical methods like MS/NMR [6, 7] and IR [8] have been described that would provide more detailed structural information on biliverdin, but these have not yet been used to study and elucidate the blue-green pigment in the fish skeleton.

The situation becomes even more confusing during the search for literature on the blue-green pigment in garfish and eelpout, where vivianite—iron(II) phosphate—is almost always the substance named [9]. The German online source Wikipedia also names vivianite as the blue-green skeletal pigment of garfish and eelpout [10, 11]. Such statements about the garfish pigment have been and

continue to be repeated in many technical publications. In fact, an official government guideline also includes the statement that vivianite in the skeleton of eelpout is the reason for the green discoloration of the bones during cooking [12]. Yet there are no scientific publications that confirm the presence of vivianite in the fish in question or that show any connection between the origin of color and increased temperature.

The purposes of this study were the following: (i) to identify conclusively the characteristic blue-green skeletal pigment in the garfish, a food fish, and in the eelpout; (ii) to conduct a quantitative estimate of the amount of pigment in the matrix; and (iii) to determine the exact location of the pigment, as a contribution to both fundamental and applied food research.

Materials and methods

Chemicals

All solvents and reagents were of analytical or HPLC grade, except for Suprapur nitric acid (65 %) and perchloric acid (70 %) from Merck (Darmstadt, Germany).

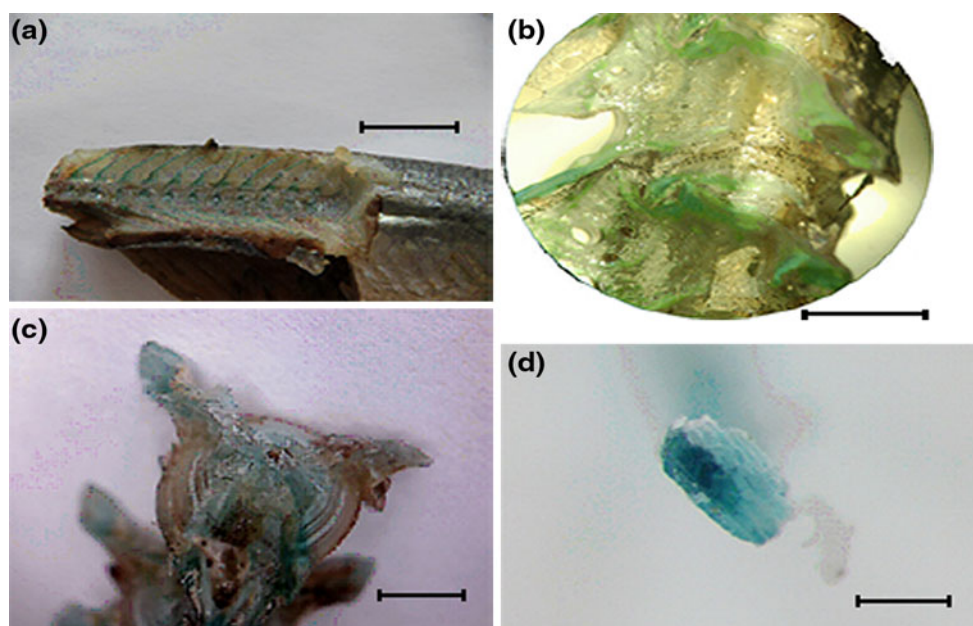
Biliverdin dimethyl ester and biliverdin hydrochloride were purchased from Frontier Scientific (Logan, UT, USA). Acetic acid (100 %), sodium chloride, L-(+)-ascorbic acid, Fe-AAS standard solution (1,000 mg/L), ammonium monovanadate, potassium chloride, potassium dihydrogenphosphate, and ammonium heptamolybdate tetrahydrate were purchased from Carl Roth (Karlsruhe, Germany); chloroform and boron trifluoride (12 % in methanol) from Acros Organics (Geel, Belgium); and methanol from Sigma-Aldrich (Taufkirchen, Germany). Water ($18 \text{ M}\Omega \times \text{cm}$) was obtained from a water purification system (Millipore, San Jose, CA, USA). The SPE columns (Chromabond C18 ec 500 mg 3 mL) were purchased from Macherey & Nagel (Düren, Germany).

Sample preparation

A ceramic knife was used to collect (dissect) the following samples of garfish and eelpout:

- The blue-green periosteum around the garfish bones of up to 0.3 mm thickness. The periosteum of the eelpout is much thinner and cannot be dissected.
- Light, white muscle tissue, which is 3 mm beneath the skin and is not colored blue-green
- Bony cartilage around the vertebral column
- Spinal processes with an intensively blue-green core (s. Fig. 1).

Fig. 1 Garfish (*B. belone*)
a Spinal and transverse processes (*bar scale 2 cm*);
b central strand of the vertebral column with blue-green layers of coating (periosteum) (*scale bar 2.2 mm*); **c** cartilage of the chordal sheath (cranial section) (*scale bar 0.5 mm*); **d** section through a spinal process with more intensive pigmentation in the center (*scale bar 0.5 mm*)



Microscopy

The samples were examined with a binocular loupe. The photographs in Fig. 1 were made with a compatible photoadapter and a reflex camera with a macro-objective.

Incineration of samples

A sample of ca. 500 mg was weighed into a 20-mL lidded Teflon beaker, mixed with 1 mL concentrated HNO_3 Suprapur, and heated for 6 h at 60 °C. After cooling to room temperature, 300 μL Suprapur perchloric acid was added, and the mixture was heated slowly (within 3 h) to 150 °C. This temperature was held for another 3 h. When the sample volume was below 600 μL and the color was still brownish, 300 μL HNO_3 and 100 μL perchloric acid were added to a total of ca. 1 mL, which was heated further at 150 °C until clear. The solution was reduced to about 300 μL and transferred into a 2-mL graduated flask and filled with 0.2 % HNO_3 to 2 mL.

Determination of iron content

The iron contents of the mineral sample solutions were determined with a Philips PU 9100X atomic absorption spectrometer from Pye Unicam Ltd. (Cambridge, England) by external calibration with a standard iron solution. In some cases the contents were confirmed by the standard addition method.

Determination of phosphate content

The phosphate contents were measured according to the determination of total phosphorus in meat and meat products according to the international standard ISO 13730:1996-12. The phosphate content of the bones was not determined because no meaningful results were to be expected, due to the high phosphate levels of the biogenic phosphate mineral apatite, which is a major component of fish bones. Dissection of the blue-green periosteum had to be undertaken with great care in order to avoid augmenting the phosphate levels due to contamination of the samples by bone abrasion.

Isolation of biliverdin (from vertebral column, bones, and periosteum)

During all steps of the process, great care had to be taken to prevent the analyte from coming into contact with atmospheric and dissolved oxygen. All relevant solutions, pipette tips, and containers were therefore stored and used where appropriate in an argon atmosphere. The analyte was protected from light as much as possible, and all procedures were performed expeditiously under dim light.

Extraction

Following a modified method of Fevery et. al. [13], ca. 1 g of sample of exactly determined weight was

suspended in a centrifuge tube with 40 mg of NaCl and 5 mL of 98 % acetic acid. Then, 10 mL of chloroform was added, followed by addition of 10 mL water. Following every solvent addition step, the mixture was homogenized for 60 s with the Ultraturrax and placed in the ultrasound bath for 5 min. Then the sample was centrifuged for 5 min at $2,500\times g$. The lower chloroform phase was transferred with a 1-mL air-displacement pipette into a weighed 25-mL pointed flask. The aqueous acetic acid phase was extracted with 5 mL chloroform two more times following the addition of 2 mL acetic acid as described above and extracted a third time with 3 mL chloroform. The combined chloroform phases were reduced under nitrogen to ca. 100 μ L and subsequently methylated.

Methylation

Following the method of Bonnet and McDonagh [7], ca. 100 mg of the chloroform extract was mixed with ca. 100 μ L of methanol to bring the total weight of the mixture to between 160 and 200 mg. A total of 2.5 times that amount of bor trifluoride solution was added and the mixture gently shaken for 20 min. The flask was then refrigerated for 2.5 h for reaction. Solid-phase extraction was conducted after derivation.

Solid-phase extraction

The C18 solid-phase extraction (SPE) tube was conditioned with 3 mL methanol and 3 mL water. The methylated sample (600–700 μ L) was mixed with seven times as much water and passed through the tube packing. The packing was washed with 3 mL water, and elution was subsequently performed with 100 μ L 0.5 % methanolic vitamin C solution and 2.5 mL methanol. The eluate was filled into 3-mL volumetric flasks.

This sample was used for UV spectroscopy, HPLC, and HPLC–MS.

For the determination of the recovery rates, ca. 100 μ L of a solution containing 90 μ g/mL of biliverdin hydrochloride was added to 1 g sample of white muscle tissue of garfish and other food fish. The same analytical steps were then carried out as for experimental samples.

UV spectroscopy

The UVIKON 930 spectrophotometer was from Bio-Tek Instruments (Neufahrn, Germany). The sample solution and methanolic solutions of biliverdin dimethyl ester and biliverdin hydrochloride were measured against methanol (300–800 nm) in 1-cm cuvettes.

FTIR

The FTIR Tensor 27, which was used with a PIKE MIRacle ATR sampling accessory, was from Bruker (Ettlingen, Germany). The peaks isolated with HPLC–UV for biliverdin dimethyl ester were collected, treated with argon, and diluted with three times as much water when sufficient pigment (ca. 1 mg) had been collected. After the solution was treated again with argon, the sample was frozen and freeze-dried. The crystals that could be scraped from the flask were analyzed in the FTIR spectrometer.

HPLC–UV

The system used for reversed-phase HPLC consisted of an HPLC pump (Maxi Star model; Knauer, Berlin, Germany) equipped with a 20- μ L injection loop, a degasser (Knauer, Berlin, Germany), and a UV detector (Variable Wavelength Monitor, Knauer, Berlin, Germany). UV detection of all peaks was performed at a wavelength of 382 nm. The HPLC separation was performed with a Eurosphere II 100-5 C18 P column with precolumn (250 \times 4 mm, Knauer, Berlin, Germany). The mobile phase was methanol/tri-distilled water (78:22, v/v) at a flow rate of 1.0 mL. Prior to use, the mobile phase was degassed by sonication for 10 min.

HPLC–MS

The HPLC system for LC/MS analysis consisted of a controller, a pump, and an autosampler equipped with an injector with a 5- μ L loop (Waters, Eschborn, Germany). The chromatographic column was a Eurochrom II 100-3 C18 P 150 \times 2 mm (5 μ m average particle size) with a precolumn. For separating the analytes, the mobile phase was methanol/water (78/22, v/v). The flow rate of the LC eluent was 0.15 mL/min.

Mass spectrometry was carried out using an LCQTM ion trap equipped with an electrospray ionization unit (Thermo Finnigan, San Jose, CA, USA) controlled by Xcalibur Software version 1.3 (Thermo Finnigan). Ionization took place in the positive ion mode by applying a potential of 4.5 V to the electrospray capillary. The capillary temperature was set at 250 °C. Sheath gas was nitrogen and auxiliary gas was helium with gas flows set at 60 and 10 U. Nitrogen was generated from pressurized air in an Ecoinert 2 ESP nitrogen generator (DWT-GmbH, Gelsenkirchen, Germany).

Ionization and mass spectrometric conditions were optimized for standard solutions of biliverdin dimethyl ester dissolved in methanol at a concentration of 5 μ g/mL. The instrument is operated in the full-scan mode over the range of 50–1,000 m/z . To obtain structural information,

collision-induced fragmentation spectra were recorded using TunePlus™ 1.3 (Thermo Finnigan) software. Collision energy values were adjusted between 35 and 40 %. After the full scan (MS), a product ion mass spectrum (MS²) for the most intense ion of the previous full scan was generated. Next, a second-order product ion mass spectrum (MS³) was generated for the most intense ion from the MS² spectrum.

Determination of amino acids

The determination of the amino acids of the periosteum, the muscles, and the spinal processes was conducted following the standard VDLUFA (Association of German Agricultural Analytic and Research Institutes) method 4.11.1 (2004) (VDLUFA-Methodenbuch III, published by VDLUFA, Darmstadt, edited by Naumann, C.; Bassler, R.; Seibold, R.; Barth, C.), and the hydroxyproline content was determined according to the International Standard ISO 3496:1994-09. In the analysis of the amino acid spectrum, cystine and methionine can only be determined incompletely/partially and tryptophan decomposes during hydrolysis.

Results and discussion

Microscopy

The microscopic view revealed blue-green pigmentation of the spinal processes (*Processus spinosus*) and the transverse processes (*Processus transversus*) (Fig. 1a). A layer of blue-green-pigmented tissue is visible on the periosteum in the area of the spinal and transverse processes. The blue-green layers are particularly heavy where the processes and ventral ribs are attached to the central strand of the vertebral column. These areas are filled with connective tissue structures rich in collagen fibers (the periosteum), which are connected to the skeletal musculature (Fig. 1b). Around the connective tissue of the chordal sheath (cranial section), colored cartilage is to be found which is connected to the layers at the root of the spinal processes (Fig. 1c). To the best of our knowledge, the blue-green coloration of this tissue has not been described in the literature on garfish and eelpout.

A cross section through the central vertebra shows only slight discoloration within, while a cross section of the rib of the spinal process shows in addition to the layering a continuous, intensive coloration and a dark blue-green core (Fig. 1d). The pterygiophores (fin rays) are also colored (not shown). In the vicinity of the dorsal fin, coloration is restricted to the proximal region, chiefly as coating, less so in the basal area (not shown).

The blue-green coloring found by Çağlar [3] in the bones of the garfish is identical to that which we observed (Fig. 1d). Çağlar described this discoloration as being found in the region of “intercellular deposits of bone salts” and speculated that the pigment probably occurs bound as a calcium salt. Our microscopic analysis showed unexpectedly that the pigment is localized not only in the bone, but to a great degree in the collagen-rich periosteum. As explained in detail below, the consistent coloration of the spinal processes can be explained by their high content of structural proteins of the same type. As Çağlar mechanically cleaned the bones before examining them microscopically, the layers of coating on the bones may have been completely removed.

Tests for vivianite

If, as indicated in the literature [9–12], the pigment in question was vivianite (Fe₃(PO₄)₂), the blue-green parts would show higher levels of Fe²⁺ and PO₄^{3−} than the normally colored parts.

Iron contents

The results of analysis of the spinal processes of the garfish and of the periosteum on the bones of the garfish and the eelpout showed that iron levels were two to three times higher than those in comparable tissues and bones of other fish, or in the white muscle tissue of garfish and eelpout (Table 1). According to Souci et al. [14], the average iron content in edible parts of fish (musculature) is 2–5 µg/g, and this corresponds to our findings for the white musculature of garfish, eelpout, and herring. Lönnberg [15] determined the Fe₂O₃ level in garfish bone ash to be 0.004 %, corresponding to an iron content of 14 µg/g, which could initially be interpreted as an indication of vivianite. However, the increased iron level in bones with a blue-green appearance and blue-green periosteum could

Table 1 Iron and phosphate contents of selected colored and non-colored fish matrices

Fish matrix	Iron		Phosphate	
	(µg/g)	n	(%)	n
Garfish, blue-green periosteum	6.6	2	0.42	4
Garfish, white musculature	3.8	4	0.39	4
Garfish, blue-green spinal processes	16.5	2	–	–
Garfish, vertebral column with periosteum	9.1	3	–	–
Eelpout, white musculature	3.7	3	0.37	3
Eelpout, blue-green periosteum	10.4	2	0.36	2
Herring, white musculature	3.97	4	0.49	4

n sample size, – not determined

also be due to an affinity of iron for biliverdin (as the cause of the blue-green color—see below).

Phosphate contents

Due to the high phosphate contents of bone, it is difficult to make statements about the determination of phosphate levels in bones, since only a fraction of the overall phosphate would be expected to occur in the pigment. On the other hand, analysis of the periosteum and muscles, which are naturally low in phosphate, should produce meaningful results. Table 1 shows these levels, calculated as phosphate, in the white musculature and periosteum of the fish investigated here. The phosphate levels in muscle tissue were found in concentrations corresponding to those of Souci et al. [14] for a variety of fish. Comparison of these phosphate levels with those found in the blue-green periosteum and white musculature shows no strikingly higher phosphate levels. If vivianite was present in the blue-green periosteum, a higher phosphate concentration would be expected in this matrix. The phosphate levels determined in the musculature and the periosteum clearly indicate that vivianite can be excluded as the cause of the blue-green color.

Altogether, the results of the analyses conducted here do not permit the conclusion that the blue-green color is due to vivianite.

Extraction of the pigment

Following indications in the scientific literature that the blue-green pigment in question could be or is biliverdin, a method was developed to extract this substance (potentially) present in the sample material. Despite the fact that methods have been published for extracting biliverdin from bile in eggshells, hardly any information is available on the recovery rates and precise amounts to be found in biological samples. Heirwegh et al. [16] reported that biliverdin is instable and can be obtained from biological samples only in small amounts and with poor precision. While the recovery rate from bile is 92.7 %, it is only 26.2 % from goose feces [17].

According to the literature, the earliest extraction procedures, for example, with hydrochloric acid and acetonitrile [17], had recovery rates of around 10 %. Recovery rates did not improve until extraction was performed with concentrated acetic acid according to Teichmann [18] and heme isolation was carried out under argon gas atmosphere with rapid extraction of the analytes in the chloroform phase. Other sources of error, such as excessive ascorbic acid amounts, corrosion of Ultra-Turrax rods, evaporation to dryness, and suction of air during solid-phase extraction, had to be dealt with and overcome by us for the present study, since these all lead to sudden color changes or even

to the formation of a colorless solution. Methylation was conducted chiefly in order to improve the chromatographic qualities of the analytes and to make our results comparable with those of other authors working on biliverdin dimethyl ester [7, 19]. Solid-phase extraction was necessary in order to remove the solvent chloroform quickly and gently and to prepare the analytes for further analysis.

Cautious and rapid extraction of the analytes from the acetic acid solution yielded recovery rates of $72.3 \% \pm 6.7$ ($n = 6$, HPLC–UV determination) with a concentration of 90 µg/g biliverdin hydrochloride in fish musculature.

The combined spectroscopic and chromatographic analyses of the samples described below indicate that the pigments studied in the skeletons of garfish and eelpout are indeed biliverdin.

Optical spectroscopy

UV–Vis

The pigment isolated from the periosteum of the garfish and methylated pigment showed UV–Vis maxima at 376 and 666 nm (Fig. 2), respectively. The reference substance biliverdin dimethyl ester shows a comparable spectrum, also with maxima at 376 and 666 nm (not shown). The maxima of the non-derivatized reference substance biliverdin hydrochloride were at 376 and 679 nm, showing that the changes in the UV spectrum due to methylation are minor. The maxima reported by Christomanos [4] in the “pure belone pigment” he isolated were at 385 and 678 nm, and these are in good agreement with our results, indicating that the pigment extracted from the periosteum of the garfish is biliverdin.

FTIR

Stretching of the ester carbonyl appeared in the IR spectrum (s. Fig. 3) at ca. $1,739 \text{ cm}^{-1}$. Lactam carbonyl absorption was observed at ca. $1,700$ and $1,675 \text{ cm}^{-1}$.

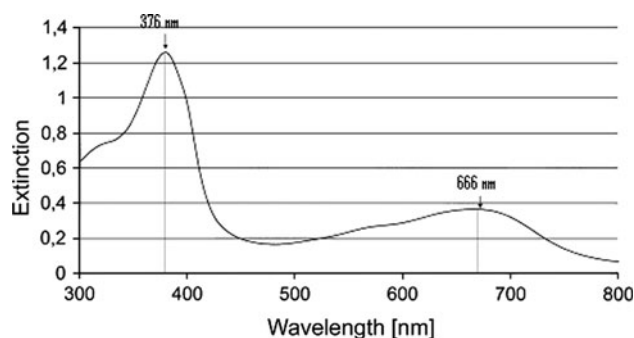


Fig. 2 UV–Vis spectrum of the blue-green pigment (biliverdin dimethyl ester) of garfish (*B. belone*) after methylation and separation

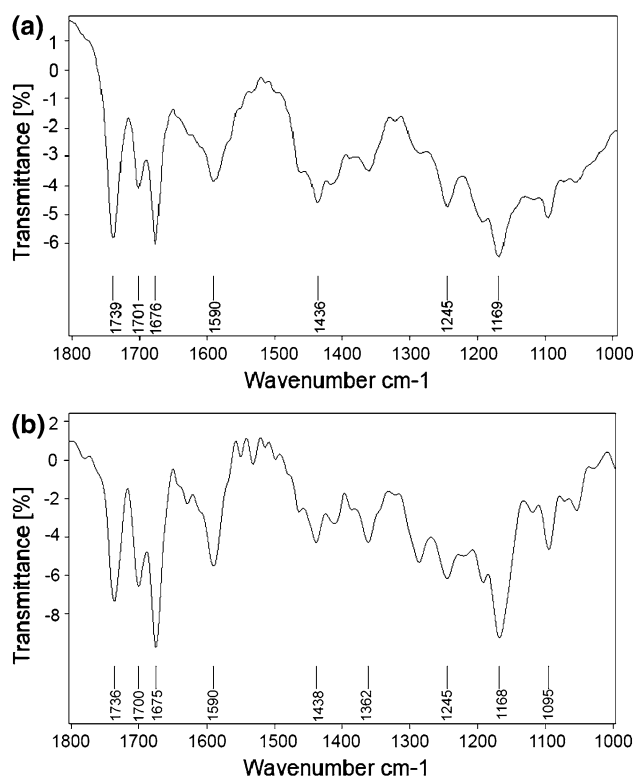


Fig. 3 FTIR spectrum of the **a** blue-green pigment (isolated by HPLC from a methylated extract of garfish (*B. belone*) periosteum and **b** the reference substance biliverdin dimethyl ester

These results are in good agreement with those for standard biliverdin dimethyl ester and with the results of Bonnett and McDonagh [7]. This spectrum is quite similar to an IR spectrum of biliverdin dimethyl ester and an UV spectrum presented in the work of Nomura and Tsuchiya [8].

HPLC–UV

Figure 4 shows the chromatogram of a sample solution prepared and methylated from garfish periosteum. Specific retention times for biliverdin, biliverdin monomethyl ester, and biliverdin dimethyl ester are evident in the chromatogram for reference substances. Under the conditions given above, biliverdin is not retarded, and the monomethyl ester has a retention time of ca. 10 min. In every case, biliverdin dimethyl ester was identified as the main component (>95 %) of the sample solutions from the colored fish parts. The HPLC investigation also permits testing of the efficiency of methylation to dimethyl ester. The identity of the extracted pigments was further confirmed by HPLC–MS.

HPLC–MS

Analysis of the methylated sample by HPLC–MS showed a mass of $m/z = 611$ characteristic of garfish and eelpout,

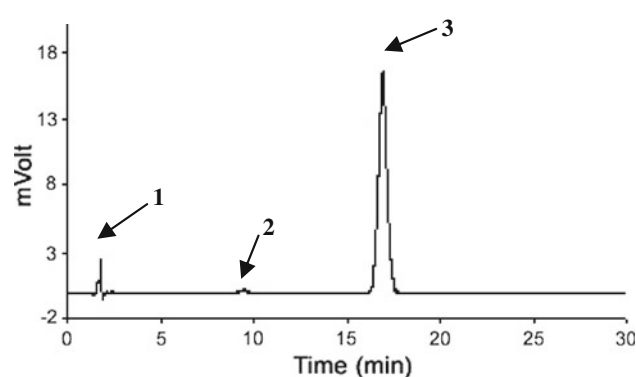


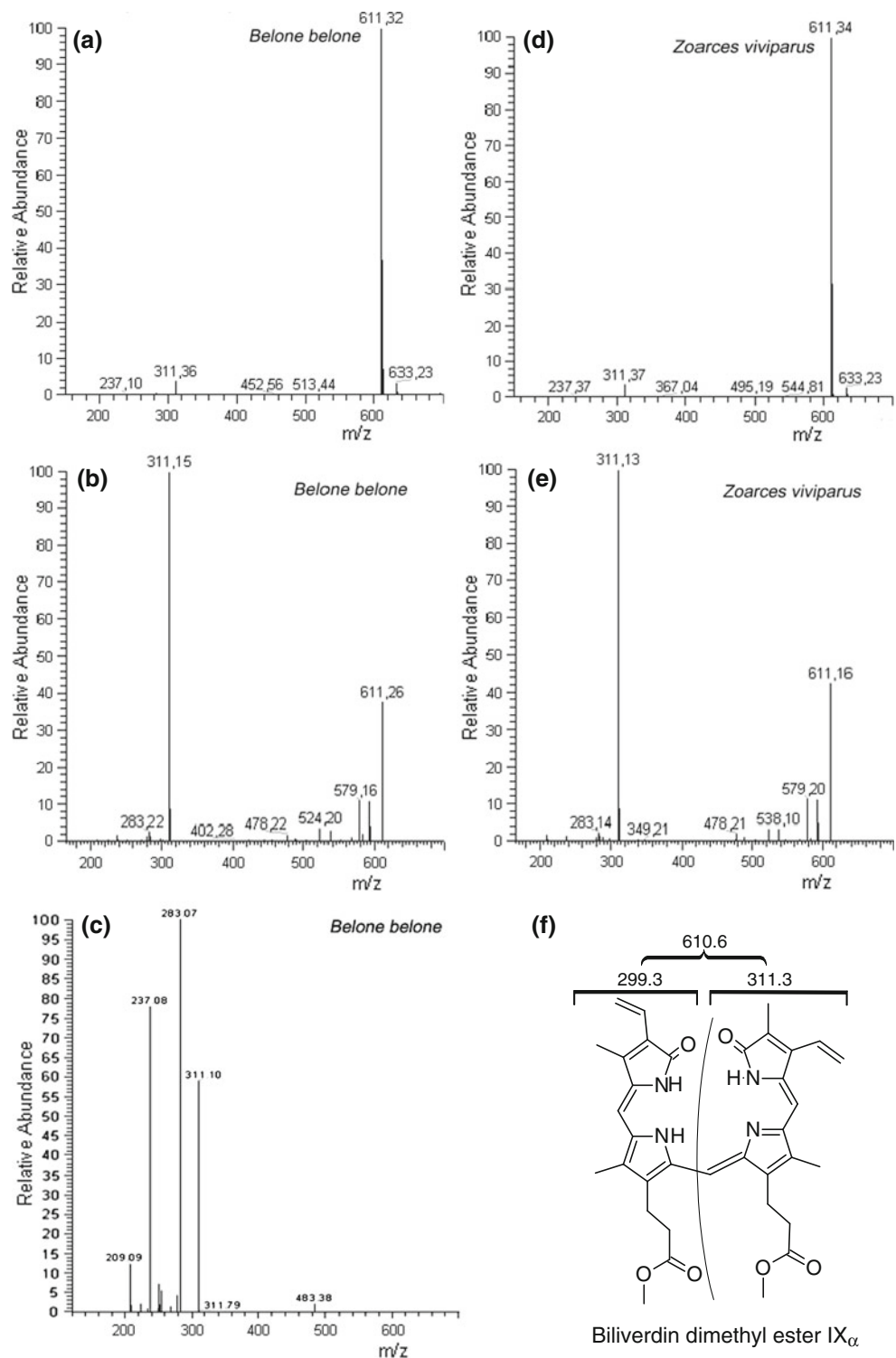
Fig. 4 HPLC chromatogram of biliverdin from garfish (*B. belone*) periosteum with the position of the biliverdin derivatives 1 biliverdin, 2 biliverdin monomethyl ester, 3 biliverdin dimethyl ester

and analysis by MS/MS, a mass of m/z 311 (Fig. 5a, b, d, e). Zhu et al. [20] conducted extensive mass spectrographic analyses of biliverdin isomers. Based on their results, the following masses are characteristic of biliverdin: m/z $[M + H]^+ = 611$ represents the molar weight plus 1, and $m/z = 311$, a fragment in MS/MS, where the biliverdin dimethyl ester is split in the middle, as in Fig. 5f. This is in accordance with the MS and MS/MS spectra of the reference substance we investigated, biliverdin dimethyl ester. In the MS^3 of the methylated garfish samples, we found m/z 283 as the main fragment; this is identical with the MS^3 identified by Zhu et al. 2005 as the non-methylated biliverdin IX α . No meaningful MS^3 could be derived for eelpout, only small amounts of which were available. Biliverdin IX α is the chief metabolite of heme. In lower animals IX β , γ , or δ metabolites also occur, which we did not detect. Overall, it can be stated that the HPLC–MS spectra of garfish and eelpout (from methylated sample solutions) correspond to a high degree with that of the reference substance biliverdin methyl ester, so that it can be assumed with certainty, and in consideration of the other spectroscopic and chromatographic analyses, that the substance in question is biliverdin.

Concentrations

Following the qualitative determination of the pigment, we determined by HPLC–UV the concentrations of biliverdin in different garfish matrices (Table 2). The highest average concentration of biliverdin (61.7 $\mu\text{g/g}$) was found in the blue-green periosteum, corresponding to the observation that there is less blue-green discoloration in the vertebral column (Fig. 1). Concentrations were lower (23.49 $\mu\text{g/g}$) in the mixture of vertebral column and blue-green periosteum. The average concentration in the spinal processes was 24.3 $\mu\text{g/g}$. This is the first quantitative report on concentrations of biliverdin in fish with blue-green skeletons.

Fig. 5 HPLC–MS of the methylated extract of blue-green matter from garfish (*B. belone*) and eelpout (*Z. viviparus*): **a/ d** MS spectrum, showing the molecular ion $[MS + H]^+ = 611$ of biliverdin dimethyl ester obtained from **a** garfish and **d** eelpout; **b/ e** MS/MS spectrum collected from molecular ion from the corresponding MS spectrum ($m/z = 611$); **c** second-order product ion mass spectrum (MS^3) collected for the most intense ion from the corresponding MS^2 spectrum ($m/z = 311$); **f** formula and the proposed fragmentation of biliverdin dimethyl ester IX_α



Localization of the pigment in the fish body

In light of microscopic observations that the blue-green pigment was chiefly to be found in the garfish periosteum, further investigations were conducted to localize it more

precisely by analyzing the amino acid composition of different samples. The results are presented in Table 3. The amino acid compositions found in the garfish periosteum and spinal processes were almost identical. Comparison with the literature [21–23] shows good agreement with the

amounts of individual amino acids in fish connective tissue from bone. Further, comparison with the amounts of the amino acids hydroxyproline and proline shows that the

Table 2 Biliverdin concentration in garfish (*B. belone*) in $\mu\text{g/g}$

White muscle tissue: not determined	< 0.1 $\mu\text{g/g}$
Spinal processes, with blue-green core	24.3 $\mu\text{g/g}$
Vertebral column + blue-green periosteum	
Sample 1	27.6 $\mu\text{g/g}$
Sample 2	15.9 $\mu\text{g/g}$
Sample 3	27.0 $\mu\text{g/g}$
Average ($n = 3$)	23.5 $\mu\text{g/g}$
Blue-green periosteum	
Sample 1	67.8 $\mu\text{g/g}$
Sample 2	55.6 $\mu\text{g/g}$
Average ($n = 2$)	61.7 $\mu\text{g/g}$

periosteum and the protein of the spinal processes chiefly consist of collagen. Analysis of the amount of hydroxyproline in the dissected blue-green garfish periosteum showed that this contained $15.0 \pm 1 \%$ connective tissue ($n = 4$) and that connective tissue proteins comprised more than 90 % of the total protein.

Comparison with the white musculature of garfish and eelpout showed an almost identical distribution of amino acids, with only traces of hydroxyproline. While the “greenish” periosteum of the eelpout contains more hydroxyproline than that of its white musculature, the thin greenish eelpout periosteum cannot be separated cleanly from the muscle, so that the samples contained large amounts of white muscle tissue. Nevertheless, the high levels of the amino acids typical of connective tissue indicate that some of the blue-green periosteum, which is rich in connective tissue, was also included in the dissected

Table 3 Amino acid compositions of selected colored and non-colored fish matrices

Matrix	Percentage of the individual amino acid in total amino acids				
	Amino acids	Garfish, blue-green periosteum	Garfish, white musculature	Garfish, spinal processes	Eelpout, blue-green periosteum
Taurine	1	1	0.3	1	1
Aspartic acid	6	10	6.2	9	10
Threonine	4	5	3.3	5	5
Serine	5	4	5	5	5
Asparagine	0	0	0	0	0
Glutamic acid	11	15	10.2	14	15
Glutamine	0	0	0	0	0
Glycine	21	6	21	11	6
Alanine	9	6	9.7	7	6
Valine	3	5	2.8	4	5
Cystine	0	1	0.2	1	1
Methionine	1	3	1.1	2	3
Isoleucine	2	5	1.9	4	4
Leucine	4	8	3.4	7	8
Tyrosine	1	3	1.1	3	3
Phenylalanine	2	4	2.5	4	4
Histidine	2	3	1.5	3	3
Carnosine	0	0	0	0	0
Tryptophan	0	0	0	0	0
Lysine	4	10	3.6	7	9
Arginine	8	6	7.7	7	7
Hydroxyproline (440 nm)	7	1	7.6	3	1
Proline (440 nm)	11	4	10.9	6	5
Total	100	100	100	100	100
Total proteins (Kjeldahl, <i>F</i> = 6.25)	16.1	15.1	21.6	13.8	15.2

The method permitted only partial analysis of the amino acids cystine and methionine and none of tryptophan, which decomposes during hydrolysis

samples. Furthermore, the literature on green and blue-green coloration in fish always refers to bones, scales, etc., but never to blue-green-colored collagen. The affinity of such coloration for collagen demonstrated here provides new approaches to the study of this distinctive physiological feature of the garfish and eelpout.

Several studies have been published on the different forms of protein binding of the biliverdin-binding proteins [24–27]. For example, biliverdin is bound to albumin in the green blood plasma of the fish, *Clinocottus analis* [24]. Yamaguchi et al. [25] reported on the biliverdin-binding proteins in the scales of the parrotfish, and Saito [26] on the biliverdin-binding protein in the molting fluid of the saturniid silkworm, *Samia cynthia ricini*, with an amino acid spectrum of the apoprotein. These two amino acid spectra are similar, but they are very different from that of collagen proteins.

Conclusion

This study makes an important contribution to fundamental and applied food research by presenting the following new findings: (i) the development of a suitable sample preparation method for the extraction of the blue-green pigment from different types of fish samples; (ii) the qualitative and quantitative determination of blue-green biliverdin in protein-rich matrices and in the skeleton of the garfish, a food fish, and eelpout; and (iii) demonstration of the affinity of biliverdin for collagen. Overall, these findings lend more precision to a scientific question posed as early as 1934 by Lönnberg [15] and which can be useful for further work in food or physiological chemistry. The partially incorrect conclusions drawn in earlier works about the characteristics of biliverdin in biological structure proteins were apparently due to the extreme (or poorly controllable) reactivity of the analytes. This work brings clarity to a long-standing topic of scientific inquiry.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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References

- Fontaine M (1941) A propos du pigment vert de l'Orphie (*Belone belone*). Bull Inst Oceanogr (Monaco) 793:1–3
- Willstaedt H (1940) Zur Kenntnis der grünen Farbstoffe von Seefischen (The green pigments of sea fish). Enzymologia 9:260–264
- Çağlar M (1950) Über das grüne Skelettpigment von *Belone belone* (On the green pigment in the skeleton of *Belone belone*). Commun FacSci Univ Ankara 3:265–280
- Christomanos AA (1957) Über die Natur des grünen Schuppenfarbstoffes der Belonen (The nature of the green pigment of *Belone* scales). Enzymologia 18:131–134
- Nomura T, Tsuchiya Y (1962) A propos des pigments bleu vert dans les écailles de certains poissons (Concerning some blue-green pigments in the scales of certain fish). Tohoku J Agric Res 12:49–53
- Rasmussen RD, Yokoyama WH, Blumenthal SG, Bergstrom DE, Ruebner BH (1980) High-performance liquid-chromatographic separation and quantification of the 4 biliverdin dimethyl ester isomers of the IX-Series. Anal Biochem 101:66–74
- Bonnett R, McDonagh AF (1973) The *meso*-reactivity of porphyrins and related compounds. Part VI. Oxidative cleavage of the haem system. The four isomeric biliverdins of the IX series. J Chem Soc Perkin Trans I 9:1881–1888
- Nomura T, Tsuchiya Y (1966) Identification du pigment bleu-vert des écailles du poisson de mer, *Cololabis saira* Brevoort (Identification of blue-green pigment of fish scales, *Cololabis saira* Brevoort). Tohoku J Agric Res 16:213–224
- Lillelund K, Terofal F (1987) Lexikon der Meeresfische. In: Teubner C (ed) Das große Buch vom Fisch. Teubner Edition, Füssen, p 43
- Gewöhnlicher Hornhecht (2012). http://de.wikipedia.org/wiki/Gewöhnlicher_Hornhecht. Accessed 21 Nov 2012
- Aalmutter (2012). <http://de.wikipedia.org/wiki/Aalmutter>. Accessed 21 Nov 2012
- Klein R, Bartel M, Paulus M, Quack M, Tarricone K, Teubner D, Wagner G (2010) Richtlinie zur Probenahme und Probenaufarbeitung Aalmutter (*Zoarces viviparus*). In: Umweltbundesamt (ed) Umweltprobenbank des Bundes. Erich Schmidt Verlag, Berlin
- Fevery J, Blanckaert N, Leroy P, Michiels R, Heirwegh KPM (1983) Analysis of bilirubins in biological fluids by extraction and thin-layer chromatography of the intact tetrapyrroles—application to bile of patients with gilbert's syndrome, hemolysis, or cholelithiasis. Hepatology 3:177–183
- Souci SW, Fachmann W, Kraut H (2008) Die Zusammensetzung der Lebensmittel—Nährwerttabellen. Wiss. Verl.-Ges, Stuttgart
- Lönnberg E (1934) De gröna benen hos näbbgäddan, *Belone*, och tånglaken, *Zoarces*. Fauna och flora 29:203–208
- Heirwegh KPM, Fevery J, Blanckaert N (1989) Chromatographic analysis and structure determination of biliverdins and bilirubins. J Chromatogr B Biomed Appl 496:1–26
- Mateo R, Castells G, Green AJ, Godoy C, Cristofol C (2004) Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography-ultraviolet detection analysis. J Chromatogr B Analyt Technol Biomed Life Sci 810:305–311
- Teichmann L (1853) Über die Kristallisation der organischen Bestandteile des Blutes. Zeitschrift für rationelle Medizin, N F 3:375–388
- Yoshinaga T, Sassa S, Kappas A (1982) A comparative study of heme degradation by NADPH-Cytochrome *c* reductase alone and by the complete heme oxygenase system - distinctive aspects of

- heme degradation by NADPH-cytochrome *c* reductase. *J Biol Chem* 257:7794–7802
20. Zhu YQ, Nikolic D, Van Breemen RB, Silverman RB (2005) Mechanism of inactivation of inducible nitric oxide synthase by amidines. Irreversible enzyme inactivation without inactivator modification. *J Am Chem Soc* 127:858–868
 21. Wang L, An XX, Yang FM, Xin ZH, Zhao LY, Hu QH (2008) Isolation and characterisation of collagens from the skin, scale and bone of deep-sea redfish (*Sebastes mentella*). *Food Chem* 108:616–623
 22. Kittiphattanabawon P, Benjakul S, Visessanguan W, Nagai T, Tanaka M (2005) Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). *Food Chem* 89:363–372
 23. Taheri A, Kenari AMA, Gildberg A, Behnam S (2009) Extraction and physicochemical characterization of greater lizardfish (*Saurida tumbil*) skin and bone gelatin. *J Food Sci* 74:E160–E165
 24. Fang LS, Bada JL (1988) A special pattern of heme catabolism in a marine fish, *Clinocottus analis*, with green blood plasma. *J Fish Biol* 33:775–780
 25. Yamaguchi K, Kubo K, Hashimoto K, Matsuura F (1977) Linkages between chromophore and apoprotein in the biliverdin-protein of the scales of big blue parrotfish, *Scarus gibbus Rüppell*. *Experientia* 33:583–584
 26. Saito H (1993) Purification and characterization of a biliverdin-binding protein from the molting fluid of the Eri Silkworm, *Samia Cynthia Ricini* (Lepidoptera: Saturniidae). *Comp Biochem Physiol B: Biochem Mol Biol* 105:473–479
 27. Rüdiger W, Abolinš L (1969) Zur Bindung von Biliverdin an das Protein im Crenilabrus-Blau (Biliverdin Binding to Crenilabrus-Blue Protein). *Experientia* 25:574–575